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THE EFFECT OF DIFFERENT OXYGEN CONCENTRATIONS ON THE RATE OF RESPIRATION OF AZOTOBACTER IN RELATION TO THE ENERGY INVOLVED IN NITROGEN FIXATION AND ASSIMILATION [1]

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INTRODUCTION

It has been shown (*7*,[3] *fig. 9*) that during nitrogen fixation by *Azotobacter* at 20 percent oxygen the reaction $C_6H_{12}O_6+60_2=6CO_2+6H_2O\triangle H=-674{,}000$ calories is one reaction that takes place at a constant rate for a considerable period of time when adequate oxygen is supplied and when the end product (carbon dioxide) is removed from the culture as fast as formed. If *Azotobacter* may be considered as a catalyst for this reaction, the rate at which the reaction proceeds (within reasonable limits) should be a function of the partial pressure of oxygen.

METHODS

Studies on the respiratory rate of *Azotobacter* at different partial pressures of oxygen, while fixing nitrogen and also while assimilating combined nitrogen, were carried out with the apparatus and technique described earlier (*7*). The different partial pressures of oxygen were obtained by filling a series of 12 tanks to 1,000 pounds pressure with a mixture of pure oxygen and nitrogen. The concentration of oxygen in each tank was then accurately determined by analysis. It was thus possible to subject the culture to any one of the 12 different partial pressures of oxygen by connecting the proper tank (*7, fig. 2,A*) to the air-conditioning apparatus.

RESULTS

RESPIRATORY RATE OF AZOTOBACTER AT DIFFERENT PARTIAL PRESSURES OF OXYGEN

While Fixing Nitrogen

Twelve hundred grams of a 36-hour culture at pH 7.2 (prepared as previously described (*7*)) was transferred aseptically to the sterile calorimeter. A gas mixture containing 17.8 percent oxygen was immediately started through the system at the rate of 14 liters per hour. The measurements were not begun, however, until the rate of respiration as measured by both the heat and CO_2 production became constant, thus insuring a constant number of living organisms. The

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[3] Italic numbers in parentheses refer to Literature Cited, p. 440.

rate of heat and CO_2 production was then studied at six different partial pressures of oxygen lower than the partial pressure of oxygen in air. At each concentration, the rate of respiration was observed continuously for 10 to 18 hours. Starting with 17.8 percent oxygen and descending to 0.5 percent, each of the six partial pressures was studied in turn. As soon as the rate of respiration with the lowest oxygen concentration was determined, the higher concentrations in an ascending order were again applied and the respiration rates were

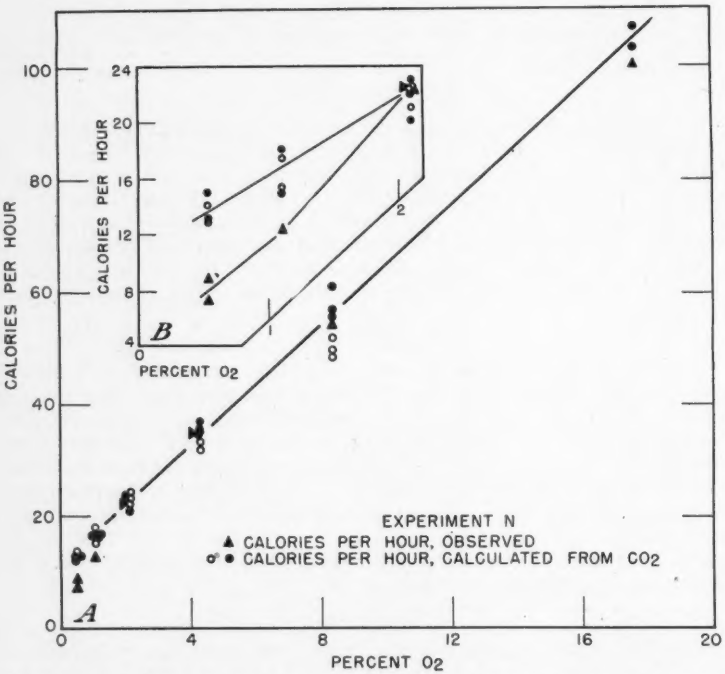

FIGURE 1.—The rate of respiration of *Azotobacter* (while fixing nitrogen) in the presence of six different partial pressures of oxygen less than that in air, applied first in a descending order and then in an ascending order. *A*, Calories per hour descending from 17.8 to 0.5 percent oxygen. *B*, Calories per hour ascending from 0.5 to 8.3 percent oxygen.

determined. In this way, the rate of respiration at each partial pressure of oxygen was approached from both extremes.

Figure 1 shows clearly the response made by the culture when subjected to the six different partial pressures of oxygen. The rate of respiration varied almost directly with the partial pressure of oxygen throughout the range studied. In the four higher concentrations less than 1 percent of the available oxygen was utilized, while in the lower concentrations the value rose to approximately 1.5 percent. Because of the enormous gas-liquid interface, and the amount of oxygen available, which was about 100 times that used, the cells did not suffer from lack of oxygen. To make sure of this, the rate of air flow was

increased 50 percent. This, however, did not increase the rate of respiration at any oxygen concentration, in spite of the 50 percent increase in available oxygen and a corresponding increase in the gas-liquid interface.

It has been shown (*7, fig. 8*) that if the rate of air flow is decreased sufficiently, depending upon the oxygen concentration, a decrease in the rate of respiration will result. However, this decrease may be due only in part to a lack of oxygen, for under such conditions carbon dioxide would accumulate in the medium, thus causing an increase in the hydrogen-ion concentration and other unfavorable conditions. When the gas-liquid interface was increased by increased rate of aeration (which would result in a more rapid gaseous exchange), a maximum rate of respiration was reached.

Figure 1, *B*, clearly shows that *Azotobacter* will respire with a respiratory quotient well above unity if the oxygen concentration is reduced below 2 percent.

The objection may be raised that the number of living organisms decreased in the same proportion as the partial pressure of oxygen was decreased. It must be remembered, however, that the total change in the rate of respiration occurred within less than 15 minutes after the partial pressure of oxygen was altered. After this sudden shift in concentration, the rate of respiration remained constant until the partial pressure of oxygen was again changed (see *7, fig. 6*). It is not reasonable to assume that the required number of organisms would die within such a short time in order to effect the observed decrease in the rate of respiration.

To demonstrate again that the number of living organisms did remain constant and were at a steady state regardless of the partial pressure of oxygen used, the rate of respiration was studied with increasing partial pressures of oxygen as described. Proceeding from the low partial pressure of oxygen to the higher, the total increase in the rate of respiration occurred within 15 minutes after the higher oxygen concentration reached the culture. Here, again, to account for this sudden increase in rate of respiration by an increase in the number of living organisms, it would be necessary to assume that the culture almost doubled its number within 15 minutes.

Twenty hours after the beginning of the experiment the rate of respiration with 8.3 percent oxygen was found to be 56 calories per hour. The rate of respiration with this same concentration of oxygen was again studied 110 hours later, under the same conditions, but after the partial pressure of oxygen had undergone nine changes. In spite of such changes in the oxygen concentration and the lapse of 110 hours, the rate of respiration was 51 calories per hour. The respiration rates at 40, 70, and 110 hours with 2.1 percent oxygen were found to be 22, 23, and 25 calories per hour respectively. Here, again, the rate of respiration is the same for a given oxygen concentration and is not affected by the previous oxygen treatment. At 50, 80, and 100 hours, the rate of respiration was 17, 17, and 15.5 calories per hour, respectively, with 1.1 percent oxygen. One hundred and thirty hours after the beginning of the experiment the final observation was made on the rate of respiration at 8.3 percent oxygen (fig. 1). Pure oxygen was immediately added and the respiratory rate was determined 45 minutes later. The rate of respiration as measured by both the CO_2 and heat production was found to increase from 56 to 245 calories

per hour. In other words, there was a 338 percent increase in the rate of respiration within 45 minutes (and very likely within 15 minutes, had readings been made) after the oxygen tension was increased from 8.3 to 100 percent. It is hardly reasonable to assume that the living

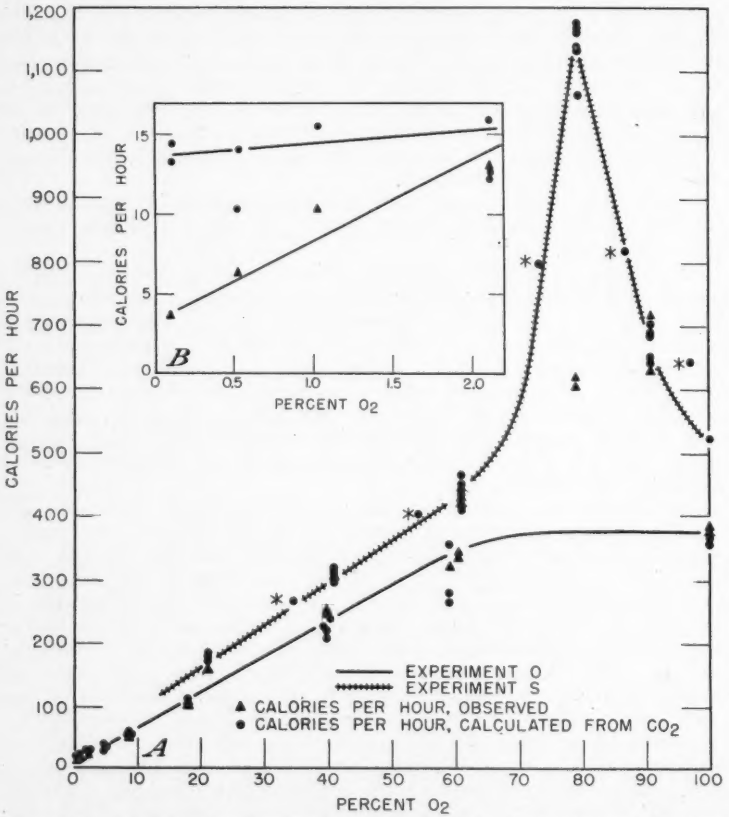

FIGURE 2.—The rate of respiration of *Azotobacter* (while fixing nitrogen) subjected to partial pressures of oxygen covering the entire range. The points designated by the asterisk represent the CO_2 evolved during 1 hour, the oxygen tension remaining at the lower value for 20 minutes and at the higher value for the remaining 40 minutes. The average tension is therefore the lower tension plus two-thirds the difference between it and the next higher oxygen tension. *A*, data from experiments O and S; *B*, the three lowest points in the data from experiment O plotted on a larger scale.

cells in the culture, subjected to so many different partial pressures of oxygen, could either increase or decrease in number in such a short time and at the same time be dependent only on the partial pressure of oxygen supplied.

To determine the effect of different oxygen concentrations above that in air on the rate of respiration and to confirm the results obtained

with partial pressures of oxygen less than that of air, the experiment was repeated. The culture was allowed to reach a steady state in 18 percent oxygen after which time the experiment was started with nitrogen containing 0.1 percent oxygen. The higher oxygen concentrations were in turn forced through the culture and the rate of respiration determined by both the heat and CO_2 produced. The results are shown in figure 2.

The results of this experiment confirm those of the previous experiment (fig. 1) over the lower range and show that the rate of respiration increases in the higher oxygen concentration up to 60 percent oxygen. Above this amount the curve appears to flatten out, but in no case does the rate of respiration decrease with increasing oxygen concentrations.

The two CO_2 points at 40 and 60 percent do not coincide with the heat measurements. It is possible that the rate of air flow was scarcely sufficient to prevent a slight accumulation of CO_2 in the medium. However, the heat measurement points fell on the curve, showing that this slight accumulation of CO_2 was not toxic. When 100 percent oxygen was added, the rate of aeration was increased from 12 to 14 liters per hour and under these conditions the rate of respiration, as calculated from the CO_2 evolved, became equal to the observed value.

To show again that the number of living organisms remained constant and that the same rate of respiration at these oxygen concentrations can be obtained by approaching from either the low or the high oxygen concentration limit, the oxygen tension was suddenly dropped from 100 to 60 percent and the rate of respiration was determined at an aeration rate of 14 liters per hour. After the effect of 60 percent oxygen was studied, 38 percent oxygen was substituted. In both cases the points fell on the curve (the highest carbon dioxide points at 40 and 60 percent oxygen). At these two points a 17 percent increase in the rate of aeration did not increase the observed rate of respiration above that obtained with the lower values for air flow. However, the calculated rate as determined by the carbon dioxide evolved was increased, but not beyond the observed rate.

Figure 2, *B*, shows the three lowest points of experiment O plotted on a larger scale. It is evident that more carbon dioxide is evolved than can be accounted for by the heat produced, which suggests that under these conditions *Azotobacter* is able to utilize combined oxygen to carry on respiration. In calculating the respiratory quotient (calories per hour calculated from the carbon dioxide evolved to the calories per hour observed) for *Azotobacter* subjected to different partial pressures of oxygen, it is evident (table 1) that in 0.1 percent oxygen this quotient is very high. A respiratory quotient of unity was not approached until the oxygen concentration had reached 2 percent.

In order to confirm the results obtained with the higher oxygen concentrations in experiment O, the experiment was repeated. In this experiment, the oxygen concentrations below that of air were omitted, but in order to define the upper portion of the curve more accurately 78.8 and 90.3 percent concentrations were also used.

TABLE 1.—*Respiratory quotient of Azotobacter while fixing nitrogen under different partial pressures of oxygen*

Oxygen (percent)	Experiment N			Experiment O		
	Average energy values per hour		Respiratory quotient (CO_2/O_2)	Average energy values per hour		Respiratory quotient (CO_2/O_2)
	Calculated from CO_2 evolved	Observed		Calculated from CO_2 evolved	Observed	
	Calories	*Calories*		*Calories*	*Calories*	
0.1				13.8	4.5	3.07
0.5	13.8	7.9	1.75	10.9	6.3	1.72
1.1	16.4	12.1	1.36	11.2	10.3	1.09
2.1	21.6	22.4	.97	13.6	13.1	1.03
4.3	35.6	34.7	1.03	34.1		
8.3	56.5	54.0	1.04	62.4	55.3	1.12
17.8	99.0	100.0	.99	105	109	.96
39.3				215	248	.87
58.8				278	322	.87
99.9				362	384	.94

The calorimeter was aseptically charged with a growing culture of *Azotobacter*, as previously described, and 21 percent oxygen was supplied until the rate of respiration reached a constant value. Each oxygen concentration was applied in turn and the rate of respiration measured continuously by both heat evolution and CO_2 absorption methods for 5 to 8 hours.

Results from experiment S, in figure 2, confirm those of experiment O and show again that the rate of respiration is dependent on the partial pressure of oxygen and that it is almost directly proportional to the oxygen concentration up to 60 percent. Both the heat produced and the heat that should have been liberated, as calculated from the CO_2 evolved, gave the same results. At 60 percent oxygen the rate of respiration continued to increase at the normal rate, while at 78 percent oxygen the rate (as measured by the CO_2 evolved) reached a maximum very abruptly. The observed rate of respiration did not increase sharply at this point.

It was further noted that at this latter oxygen concentration, only 53 percent of the energy liberated, as calculated from the CO_2 evolved, was found as heat. That this represents a critical point is evident from the fact that the rate of respiration remained constant at 78 percent oxygen for 4½ hours. The oxygen concentration was greater than 60 and lower than 90 percent for 6½ hours, as shown by eight CO_2 determinations. The points designated by the asterisk represent the rate of respiration due to the slight mixing of the two gas mixtures in the apparatus when the oxygen concentration was increased to the next higher value. At 90 percent oxygen all the energy liberated, as calculated from the evolved CO_2, was again found as heat. With but one CO_2 measurement and no heat measurements at 100 percent oxygen the rate of respiration at this concentration is uncertain in this experiment.

It is difficult to explain the sudden increase in respiration as shown by the CO_2 evolved at 78 percent oxygen. It is interesting to note, however, that the oxygen-nitrogen ratio (78 percent oxygen) which gave the maximum rate of respiration is the same as that in the nitrate ion. A further discussion will be presented later.

To demonstrate that a maximum does exist at 78 percent oxygen, the experiment was carefully repeated a month later, care being taken to remove completely the carbon dioxide from the culture by increasing the rate of aeration as the rate of respiration increased. The results are shown in figure 3, experiment T. Here, as in experiment S

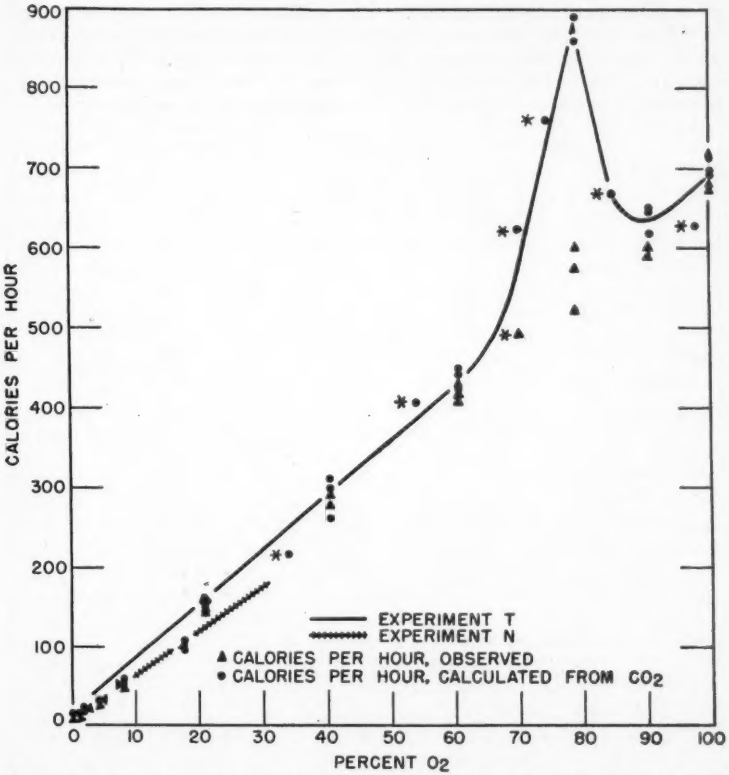

FIGURE 3.—The rate of respiration of *Azotobacter* (while fixing nitrogen) subjected to oxygen concentrations from 20 to 100 percent. The points designated by the asterisk represent the carbon dioxide evolved during 1 hour, the oxygen tension remaining at the lower value for 20 minutes and at the higher value for 40 minutes. The average oxygen tension is therefore the lower tension plus two-thirds the difference between it and the next higher oxygen tension.

(fig. 2), a maximum calculated rate of heat production occurred at 78 percent oxygen. This experiment also shows that the rate of respiration at 100 percent oxygen is slightly greater than at 90 percent. Experiment N is shown (fig. 3) in order that the slopes of the two curves may be compared.

An experiment was conducted in order to make certain that the culture was fixing nitrogen throughout the entire time the experiments were being conducted. One liter of sterile medium was transferred to a Dewar flask which was submerged in a water bath at 25° C. The

nitrogen-free medium was inoculated with 10 cc. of inoculum from the same source as that used in the other experiments. The culture was vigorously aerated with preconditioned air in a manner similar to that described earlier (*7*).

An indication of the heat produced by the culture was obtained by measuring the rise in temperature inside the Dewar flask with the aid of a Beckmann thermometer. The temperature of the water bath, in which the culture was submerged, was increased at the same rate as the temperature increased inside the Dewar flask. By this means the heat leak of the culture was reduced to a negligible amount. The amount of carbon dioxide evolved and the rise in temperature were

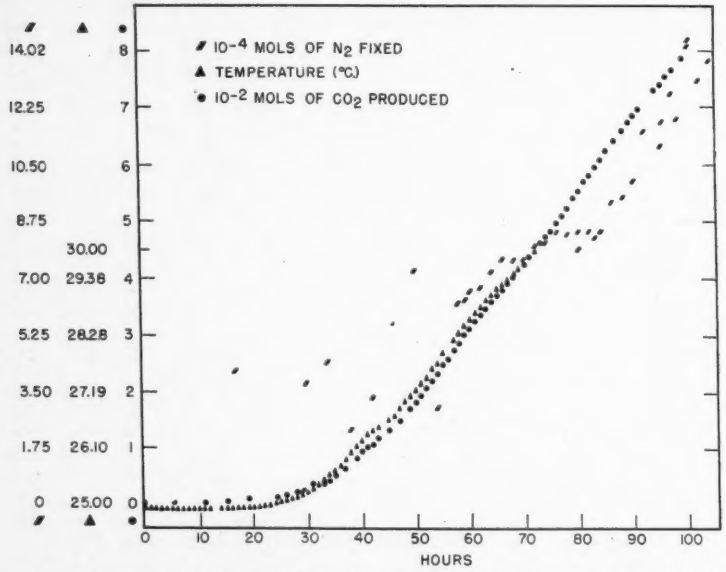

FIGURE 4.—The rise in temperature, the amount of nitrogen fixed, and the CO_2 evolved by a culture of *Azotobacter* over a period of 100 hours.

determined at regular intervals. Samples of the culture were removed at intervals and the total nitrogen determined by a standard method. The results are shown in figure 4.

It is evident that nitrogen fixation took place throughout the entire 100-hour period. It appears that after the rate of carbon dioxide production reached a constant value, the rate of nitrogen fixation also appeared to be constant and was closely correlated with the rise in temperature and the rate at which carbon dioxide was liberated.

Concerning the maximum respiration rate at 0.8 atmosphere obtained by the author (figs. 2 and 3), Lineweaver, Burk, and Horner (*11, p. 503*) state:

> * * * The value of maximum respiration at 0.8 atmosphere O_2 reported by Fife * * *[4] was obtained under conditions where oxygen pressure was not the only variable. The sugar concentration decreased considerably (about 10

[4] In Science 73:533. 1931.

gm. per liter) during the course of the experiment, and the rate of gas flow was increased at the higher oxygen pressures (private communication). The ratio of gas-liquid interface to volume of solution (as distinguished from the far less important ratio of volume of gas to volume of liquid) was critically low and any resulting lessened removal of CO_2 or establishing of respective equilibrium concentrations of O_2 (at different O_2 pressures) in all local parts of the culture medium would result in a *raised* maximum, not a lowered maximum as Fife believed.

This criticism, in the opinion of the writer, is not only in error, but is also unwarranted for several reasons. (1) the oxygen pressure was not a variable. In all experiments, the gas mixtures were supplied from tanks as described (7). It is obvious that the oxygen pressure remained constant at all partial pressures of oxygen studied. (2) The sugar concentration did not decrease about 10 gm. per liter as stated. The total amount of sugar present was only 10 gm. In experiment S (fig. 2), in which the maximum amount of sugar was consumed, only 50 percent was liberated as carbon dioxide. In the experiments in which combined nitrogen was supplied to the culture, only 24 percent of the available sugar was consumed. It seems logical from the mass law that such a decrease in this reactant would result in a decrease in the rate of respiration if the critical concentration was approached. (3) It is true that the rate of aeration was increased at the higher partial pressures of oxygen (7, *fig. 8*). However, a 67 percent increase in the rate of aeration above 14 liters per hour did not increase the rate of respiration. It appears that Burk and associates failed to appreciate the enormous gas-liquid interface supplied by rapid aeration with small bubbles of gas which rise through the liquid to the surface of the culture. A detailed discussion of this enormous gas-liquid interface available by rapid aeration is found elsewhere (see 7, *p.* 235). It may be stated, however, that at the maximum rate of respiration (1,160 calories per hour) 231.5 cc. of oxygen was utilized by the 1,200 cc. of medium, which was only 1.5 percent of the total oxygen available. At this rate of respiration each cubic centimeter of medium absorbed 0.195 cc. of oxygen per hour. The total gas-liquid interface exposed to the culture over a period of 1 hour was 210,000 $cm.^2$; that is to say, each cubic centimeter of medium had 175 $cm.^2$ of gas-liquid interface from which to absorb 0.195 cc. of oxygen in 1 hour. This does not include the interface at the surface of the medium. (4) Equilibrium was established within a few minutes upon changing the partial pressure of oxygen to either higher or lower values. This has been demonstrated (7, *fig. 6.*)

While Assimilating the Ammonium Ion

To determine whether or not the sudden increase in respiration, as measured by CO_2 produced at 78 percent oxygen, is associated with nitrogen fixation, experiments were carried out in which the culture was supplied with combined nitrogen and the rate of respiration was studied over the entire range of oxygen pressures as before. In these experiments the ammonium ion was added to prevent fixation of atmospheric nitrogen. The medium was the same as that previously used, except that 200 mg. of nitrogen as ammonium sulfate was added. The medium was inoculated and aerated as before and then transferred aseptically to the sterile calorimeter. Aeration was at once begun with 20 percent oxygen and continued until the rate of heat and CO_2 production became constant.

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The experiment was then begun by aerating with 0.1 percent oxygen at the rate of 15.7 liters per hour. One percent oxygen was next added, and from then on the oxygen concentrations were increased until the culture was subjected to pure oxygen. As the rate of CO_2 production increased, the gas-liquid interface was increased by increasing the rate of aeration, in order to maintain the CO_2 concentration in the medium at a minimum. The air flow was increased from 15.7 to 17.5 liters per hour when 40 percent oxygen was added, and at 60 percent oxygen it was increased to 18 liters per hour. When 80 and 100 percent oxygen was added, the rate of air flow was increased to 19.5 liters per hour. The effect of different oxygen con-

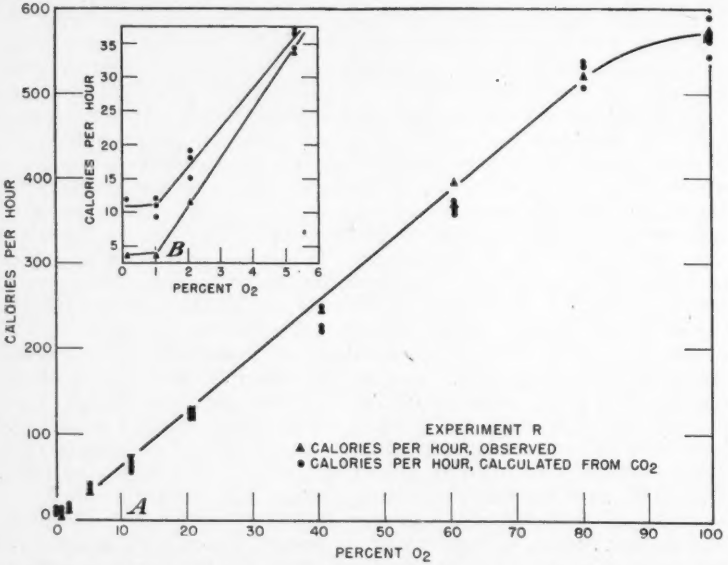

FIGURE 5.—*A*, The rate of respiration of *Azotobacter* (while assimilating the ammonium ion) under oxygen concentrations from 0.1 to 100 percent; *B*, data for low oxygen concentrations plotted on an enlarged scale.

centrations on the rate of respiration, when the ammonium ion was assimilated, is summarized in figure 5.

Apparently the rate of air flow was insufficient at 40 percent to remove the carbon dioxide, in spite of the increased rate of air flow. The increase made at 60 percent improved conditions, but not until the air flow was increased to 19.5 liters per hour at 80 percent oxygen was it sufficient to remove the carbon dioxide effectively. Here the rate of respiration is almost directly proportional to the partial pressure of oxygen between 2 and 80 percent. From 80 to 100 percent oxygen, the rate of respiration continued to increase but at a slower rate. It will be noticed that the curve also flattens out below 2 percent oxygen (*fig. 5, B*), thus showing that at these low concentrations the rate of respiration is not affected by a further decrease in

the partial pressure of oxygen. The curve also shows the extent to which intramolecular respiration is taking place.

Column 4 of table 2 shows that the respiratory quotient of *Azotobacter* did not reach unity until an oxygen concentration of 5 percent was reached. It appears that *Azotobacter* is able to catalyze fermentation reactions to a considerable extent at the lower oxygen pressures when the ammonium ion is present and even to a small degree in concentrations of oxygen as high as 5 percent. No sudden increase in the rate of respiration at 78 percent oxygen was obtained when combined nitrogen in the form of the ammonium ion was supplied to the culture.

TABLE 2.—*Respiratory quotient of a culture of Azotobacter while assimilating the ammonium ion under different partial pressures of oxygen*

Oxygen (percent)	Average energy values per hour		Respiratory quotient (CO_2/O_2)	Oxygen (percent)	Average energy values per hour		Respiratory quotient (CO_2/O_2)
	Calculated from CO_2 evolved	Observed			Calculated from CO_2 evolved	Observed	
	Calories	*Calories*			*Calories*	*Calories*	
0.1	12.4	3.5	3.54	20.4	123	120	1.01
1.0	10.7	3.5	3.09	40.3	228	244	.94
2.1	16.8	11.5	1.46	60.8	364	370	.98
5.3	38.8	33.5	1.16	80.7	521	522	1.00
11.5	63.3	71.6	.89	99.9	566	575	.98

While Assimilating the Nitrate Ion

To study the rate of respiration of a culture of *Azotobacter* while in the process of assimilating the nitrate ion, the above experiment was carefully repeated except that the medium contained 200 mg. of nitrate nitrogen instead of the ammonium ion. This medium had a pH of 7.1 as compared to 7.2 for the ammonia experiment. The rate of air flow was 14 liters per hour for the lower oxygen concentrations. At 40 percent oxygen the air flow was increased to 15.7 liters per hour; at 100 percent, to 17.8 liters. This experiment is summarized in figure 6.

From 10 percent to pure oxygen the rate of respiration was directly proportional to the partial pressure of oxygen, as measured both by the heat produced and the carbon dioxide evolved. Owing to the clogging of the cooling coil inside the calorimeter it was impossible to make heat measurements and thereby confirm the carbon dioxide measurements at 100 percent oxygen.

It is quite evident from fig. 6, *B*, and table 3 that anaerobic dissimilation and probably nitrate reduction takes place to a large extent below 5 percent oxygen. The respiratory quotient decreased from 5 at 1 percent to 0.97 at 11.5 percent oxygen. It is evident that the high respiratory quotients cannot be ascribed to a lack of atmospheric oxygen, since less than 1 percent of the available oxygen was utilized. The fact that the rate of respiration of *Azotobacter* was greater in the presence of the nitrate ion than when the ammonium ion was available indicates that the nitrate ion may be preferred.

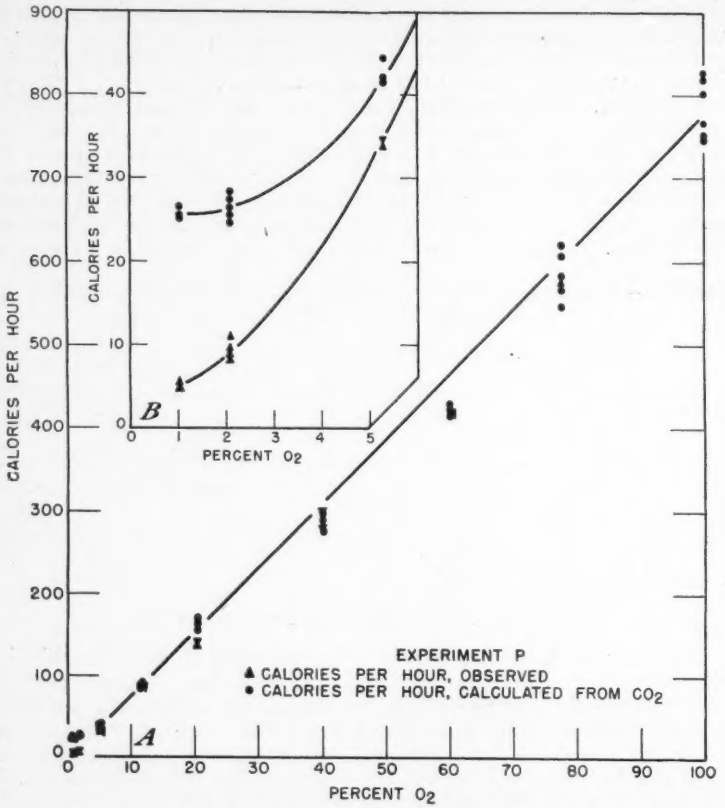

FIGURE 6.—*A*, the rate of respiration of *Azotobacter* (while assimilating the nitrate ion) under oxygen concentrations ranging from 1 to 100 percent; *B*, the data for low oxygen concentrations plotted on an enlarged scale.

TABLE 3.—*Respiratory quotients of Azotobacter obtained while the culture was assimilating the nitrate ion under increasing partial pressures of oxygen*

Oxygen (percent)	Average energy values per hour		Respiratory quotient (CO_2/O_2)	Oxygen (percent)	Average energy values per hour		Respiratory quotient (CO_2/O_2)
	Calculated from CO_2 evolved	Observed			Calculated from CO_2 evolved	Observed	
	Calories	*Calories*			*Calories*	*Calories*	
1.0	25.3	5.1	5.0	40.0	280	290	0.97
2.1	26.4	8.8	3.0	60.0	422	420	1.00
5.3	42.7	33.5	1.3	77.2	598	580	1.03
11.5	87.2	90.0	.97	99.9	833		
20.4	156	145	1.07				

CHANGE IN THE RESPIRATORY QUOTIENT WITH DECREASING PARTIAL PRESSURES OF OXYGEN

Since the publication of the free-energy data by Lewis and Randall (*9*), attempts have been made to determine the efficiency of nitrogen fixation by *Azotobacter* at different oxygen concentrations. It must be remembered that the free energy liberated by intramolecular reactions is as available to the cell as that liberated by the consumption of free oxygen, provided the reaction releasing such free energy is a step in the series of reactions which are vital to the cell. In view of this, the

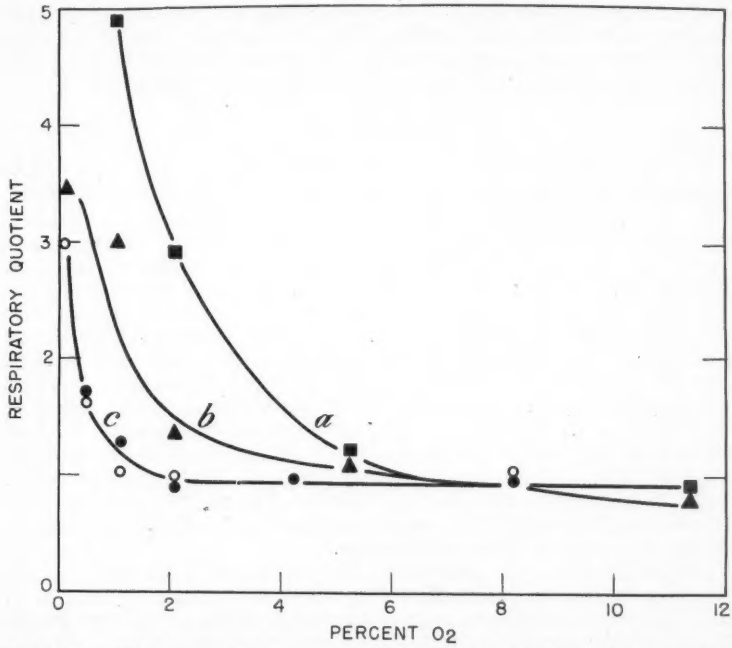

FIGURE 7.—The increase in intramolecular respiration with decreasing concentrations of oxygen by *Azotobacter* during nitrogen fixation and nitrogen assimilation: *a*, Nitrate-ion assimilation; *b*, ammonium-ion assimilation; *c*, nitrogen fixation.

energy liberated by intramolecular reactions must be taken into account before the efficiency of any cell process can be determined.

The data in figure 7 are presented to show how the respiratory quotient of *Azotobacter* increases during nitrogen fixation and nitrogen assimilation with decreasing oxygen concentrations.

It is evident from figure 7, *a* and *b*, that anaerobic dissimilation (reactions involving combined oxygen) is taking place to a marked degree by *Azotobacter* while assimilating either the nitrate or the ammonium ion. At partial pressures of oxygen greater than 5 percent, these reactions were not detectable. At this and lower values, especially in the presence of the nitrate ion, *Azotobacter* utilizes large amounts of combined oxygen with the production of carbon dioxide.

The curve clearly shows the ease with which *Azotobacter* is able to utilize combined oxygen for respiration in the presence of the nitrate ion and at low oxygen pressures where free oxygen is not readily available.

When *Azotobacter* was forced to fix atmospheric nitrogen (fig. 7, *c*) it is evident that a lower partial pressure of oxygen was necessary to force the cells to catalyze intramolecular reactions than when combined nitrogen was present. In two separate experiments the respiratory quotient was found to remain at unity until the partial pressure of oxygen was reduced to 2 percent. Below this concentration, however, the respiratory quotient increased rapidly until at 0.1 percent oxygen, where it reached a value of 3. It is clearly evident that the free oxygen consumed by *Azotobacter* at partial pressures below 2 percent is by no means a measure of the total energy liberated.

DISCUSSION

The data presented here and in previous papers (*6*, *7*) are not in agreement with those of other investigators. Meyerhof and Burk (*12*) and Burk and associates (*2*, *3*, *4*), Lineweaver (*10*), and Lineweaver and associates (*11*) have presented considerable data on the physiology of *Azotobacter* with special reference to the efficiency of nitrogen fixation and efficiency of growth. In these experiments the Warburg (*13*) apparatus was used exclusively for measuring the rate of respiration (free oxygen consumed). The efficiency of nitrogen fixation and the efficiency of growth of *Azotobacter* were determined over the entire range of oxygen pressures, with the Warburg apparatus and technique (*13*). A critical discussion of this has been given by the author (*7*). Meyerhof and Burk (*12*) report that the rate of respiration increased with increasing oxygen pressure until 15 to 20 percent oxygen was reached. Above this concentration, they found that the respiration rate fell off rapidly. The experiments of these investigators show that the rate of respiration in pure oxygen is from one-third to one-half the value for air.

The author has failed to confirm the findings of these workers. In 10 experiments (examples of which are shown in figures 1, 2, 3, 5, and 6) more than 100 separate measurements on the rate of respiration of *Azotobacter* were made, in which five concentrations of oxygen greater than that in air were used. Each of these separate measurements (eight examples of which are shown in detail in figure 6 of an earlier paper) (*7*) consisted of measuring simultaneously the heat and carbon dioxide produced over a period of at least 2 hours. In every case the author found the rate of respiration of *Azotobacter* in concentrations of oxygen above 20 percent to be greater than at 20 percent.

The duration of experiments S (fig. 2) and T (fig. 3) was 38 and 36 hours, respectively. By making the heat and carbon dioxide measurements continuously for the entire time in each experiment, any decrease in the rate of respiration would surely have been detected. Continuous heat and carbon dioxide measurements were also made in experiments R (fig. 5) and P (fig. 6) which were of 80 and 140 hours duration, respectively, with no indication of a decrease in the rate of respiration at the higher oxygen concentrations.

It is difficult to see how equilibrium between the gas and liquid

phases could have been reached and maintained with the apparatus used by Meyerhof and Burk, in the light of the data presented by the author, which demonstrates the precautions necessary to supply the culture with adequate oxygen. It is doubtful whether the gas exchange could proceed rapidly enough at the higher oxygen pressures in the Warburg apparatus to allow respiration to take place at a maximum rate. In view of the fact that the total volume of their apparatus was only 12 cc., convection currents in both the gas and liquid phase would be very small. Circulation of the gas would also be reduced to a minimum owing to the shape of the apparatus and the constant temperature at which it was maintained. It is true that the whole apparatus was rocked back and forth at a constant rate. However, this rate (120 cycles per minute, according to observations made by the author) was probably not rapid enough to break the surface of the liquid and by actual measurements the liquid was raised approximately 2 mm. on the side of a vessel similar to that used by Meyerhof and Burk (*12*) when the rate of shaking was 120 cycles per minute at an amplitude of 3 cm.

A striking example of the need for vigorous stirring was clearly demonstrated in an earlier paper (*7, fig. 9*). The points designated by the asterisk were obtained while the mechanical stirrer was idle. Although the culture was being vigorously aerated at the rate of 14 liters per hour, the stirring by aeration alone was only sufficient to produce a rate of respiration one-half of the maximum. In spite of such a high rate of aeration and a large gas-liquid interface, vigorous mechanical stirring was found to be indispensable for the cells to respire at a maximum rate.

Meyerhof and Burk (*12*) apparently assumed that the amounts of oxygen consumed were too small to alter the concentration of the gas phase. By actual measurement the author found that a maximum of 0.21 cc. of oxygen was consumed per cubic centimeter of medium per hour. It may well be that in their experiments the carbon dioxide was not removed from the medium rapidly enough to insure the most rapid rate of respiration, especially at the higher oxygen concentrations. As the pH of the medium used by these investigators was 7.3, this alone would retard the carbon dioxide from diffusing from the medium into the atmosphere above, at least until the concentration of carbon dioxide in the medium became appreciable. Moreover, the concentration of carbon dioxide necessary to become toxic would be reached much sooner at the higher oxygen pressures, due to increased rate of respiration. As a consequence, the rate of respiration would probably fall off sharply.

In the experiments performed by Meyerhof and Burk the ratio of carbon dioxide-free air to the volume of the culture was about 6 to 1 and the air was not bubbled through the culture medium. The author found it difficult to remove effectively the carbon dioxide at the higher oxygen concentrations when the carbon dioxide-free air bubbled through the well mechanically stirred medium (pH 7.2). This was found to be true even when the ratio of carbon dioxide-free air to the volume of medium was 15 to 1. As a matter of fact, in some experiments this ratio had to be increased to 18 to 1 (with corresponding increase in the gas-liquid interface) before the carbon dioxide was effectively removed. The necessity of vigorous aeration for securing a maximum rate of respiration is seen clearly when it is realized that

the 18 liter per hour rate completely replaced and swept out all the gas in the calorimeter once every minute.

It appears that the Warburg apparatus as used by Meyerhof and Burk (*12*) and Burk (*2, 3*) and Burk and Lineweaver (*4*) is probably unreliable and incapable of effecting the very rapid and large gas exchange that is necessary if *Azotobacter* is to respire at a maximum rate at the higher oxygen pressures. Hunter (*8*) found that the maximum growth rate for *Azotobacter* could be obtained only under conditions of vigorous aeration.

Meyerhof and Burk (*12*) and Burk (*3*) concluded from certain of their experiments that the nitrogen fixation process is more efficient (less energy is expended per milligram of nitrogen fixed) at the lower oxygen pressures. They state that the efficiency is 10 to 20 times greater at 0.1 percent oxygen than in air. These investigators prepared the experimental organisms by first growing them for 24 to 48 hours in 250-cc. gas wash bottles containing 50 cc. of culture medium. During this period the cultures were aerated with compressed air (21 percent oxygen) at the rate of 100 cc. per minute. After proper dilution with sterile media to obtain the desired number of organisms, 3 to 5 cc. of this culture was transferred to the respiration vessel. It is important to point out that the cells and medium have been aerated and in contact with air containing the normal amount of oxygen. Consequently, the cells and media are saturated with oxygen at a pressure equal to that in air.

In order to study the efficiency of nitrogen fixation at 0.1 percent oxygen these workers passed this gas mixture down through the manometer stopcock and out of the ground-glass opening in the neck of the side cup of the reaction vessel at the rate of 1 to 2 liters per minute for 2 or 3 minutes. The stopcock and side cup were then closed simultaneously. At the maximum these investigators relied on 6 liters of gas flowing through the apparatus to sweep all the air from such an irregular-shaped vessel. At the same time they attempted to establish a gas mixture of 0.1 percent oxygen and to reduce the oxygen concentration of the culture medium from 20 in one case to 0.12 percent oxygen in 2 to 3 minutes without the gas bubbling through the medium.

It is difficult to see how the system set up by the above investigators could be brought to a steady state with respect to the gas-medium phase, especially where a 200-fold oxygen-pressure change was involved, with only 6 liters of gas passing over the medium in the course of 2 to 3 minutes. The data presented by the author (*7, fig. 7*) show that it required from 8 to 14 hours for no less than 160 liters of gas to bubble through the well-stirred medium. It appears from the data presented here that the experiments of Meyerhof and Burk on the efficiency of nitrogen fixation were completed before equilibrium was reached at the lower partial pressures of oxygen.

It must be remembered that it is not the partial pressure of oxygen over or in the culture medium that ultimately controls the rate of respiration, but the partial pressure of oxygen within the living cells where the reaction is taking place. It is the concentration of oxygen at this point that must be reduced from 20 to 0.1 percent before it can be said that the organisms are respiring under the influence of the lower gas mixture.

Meyerhof and Burk (*12, p. 139*) present a curve the data for which

were obtained by the above-described technique. The data show an increase in efficiency of nitrogen fixation with decreasing oxygen pressures. These investigators point out from this curve that the efficiency of nitrogen fixation increases from approximately 1 percent at 0.20 atmosphere to approximately 4 percent at 0.02 atmosphere of oxygen. At this point the slope of the curve is shown to increase rapidly as the partial pressure of oxygen approaches zero. Under these same conditions, the efficiency of nitrogen fixation is shown to approach a value of 12 percent.

When studying the energy relations of a biological process at the lower partial pressures of oxygen, where intramolecular respiration is possible, a measure of only the free oxygen consumed would be unreliable and misleading. If an organism is catalyzing several energy-yielding reactions in which combined oxygen and free oxygen are utilized simultaneously, it is evident that a measurement of only the free oxygen consumed would be of little value in determining the total energy liberated. Similarly, if only the carbon dioxide evolved were measured in such a case, it too would fail to point out the true energy relations.

As these investigators based their entire measurements on the free oxygen absorbed, it is only natural that they should observe increased efficiencies of nitrogen fixation at the very low oxygen pressures because of intramolecular respiration. (See figure 7 for the extent to which intramolecular respiration takes place while both nitrogen fixation and nitrogen assimilation are taking place.)

The conclusions drawn by Meyerhof and Burk (*12*) on the efficiency of nitrogen fixation, and also by Burk and Lineweaver (*4*) on the efficiency of growth at the lower oxygen pressures are therefore questionable for two reasons: (1) When partial pressures of oxygen lower than 5 percent were used, true equilibrium was probably not obtained; that is, the oxygen absorbed by the medium and the cells was not completely removed and a steady state established at the lower oxygen value before the experiments were begun. No data were presented on this point. (2) Meyerhof and Burk did not attempt to measure the intramolecular respiration that took place. It must be admitted that conditions are extremely favorable at such low pressures of oxygen for the culture to catalyze such reactions and the free energy liberated by them must be considered in the efficiency of the nitrogen fixation process and the efficiency of growth.

That *Azotobacter* does catalyze reactions not involving free oxygen with the liberation of energy cannot be doubted in the light of Bonazzi's (*1*) experiments which have been well confirmed by the author. The extent to which such reactions take place is brought out in tables 1, 2, 3; in *B* of figures 1, 2, 5, and 6; and in figure 7. When the culture was forced to fix nitrogen, it is seen that at 0.1 percent oxygen only one-third of the carbon dioxide produced could be accounted for by the heat evolved. In other words, at 0.1 percent oxygen, respiration with free oxygen was but one-third of the total respiration that took place.

From table 1, it is seen that the gas mixture must contain at least 2 percent oxygen before reactions involving combined oxygen are reduced to a point at which they are negligible. When *Azotobacter* was respiring in the presence of combined nitrogen, the experiments show that it required considerably higher concentrations of oxygen to

reduce intramolecular respiration to a minimum. It is evident that the amount of energy liberated by intramolecular reactions is appreciable, in proportion to the total amount liberated, at the low oxygen pressures.

There are several points of striking interest in the data presented which may shed some light on the mechanism of nitrogen fixation. It was shown in a previous paper (*6*) that the sudden increase in the rate of respiration during nitrogen fixation, as measured by the carbon dioxide evolved, occurred at the same oxygen-nitrogen ratio (78 percent oxygen) as found in the nitrate ion. At this oxygen value (figs. 2 and 3) the heat produced was only 62 and 52 percent of that calculated from the carbon dioxide evolved for the two experiments reported. That this fact is significant is evident when it is recalled that no maximum rate of respiration as measured by the carbon dioxide evolved occurred at this oxygen tension when nitrogen in the form of the ammonium or the nitrate ion was supplied to the culture.

In every case in which nitrogen in a combined form was supplied to the culture the heat measured was equal to that calculated from the carbon dioxide evolved in the regions where intramolecular respiration was absent.

It is interesting to note that the increase in rate of respiration per unit increase in the partial pressure of oxygen is greatest when the culture is assimilating the nitrate ion. Here the rate of respiration is directly proportional to the partial pressure of oxygen. This ratio of unity remained constant over the entire oxygen range above 5 percent. It required a greater concentration of oxygen to prevent intramolecular respiration when the culture was assimilating the nitrate ion than when nitrogen was supplied as an ammonium salt or by nitrogen fixation.

Bonazzi (*1*), on recalculation of data published by Hills, pointed out that *Azotobacter* is able to liberate nitrogen by utilizing the oxygen from the nitrate ion and that the nitrate ion is used by *Azotobacter* in preference to the ammonium ion.

The data presented here show that the metabolic activities of *Azotobacter* are greater in the presence of the nitrate ion than in the presence of the ammonium ion. This would also indicate a preference for the nitrate ion by *Azotobacter*.

If Bonazzi's interpretation of data published by Hills is correct, it would appear that *Azotobacter* is capable of catalyzing the reaction $2NO_3 \rightarrow N_2 + 3O_2$.

The fact that below 5.0 percent oxygen *Azotobacter* catalyzes certain reactions not involving free oxygen (this is shown by the fact that the heat evolved is much less than that calculated from the carbon dioxide produced (fig. 6)) may also indicate that oxygen is being taken from the nitrate ion. If the oxygen is not taken from the nitrate ion then the presence of the nitrate ion is unique in that it is instrumental in causing the oxygen to be removed from other compounds and liberated as carbon dioxide. From these considerations it is not unreasonable to suppose that *Azotobacter* may catalyze the above reaction in either direction.

Several writers have suggested that the first product of nitrogen fixation by *Azotobacter* is ammonia. There is, however, no conclusive

evidence to support this contention. Burk and Horner *(5 p. 121)* state that—

> the mere occurrence of ammonia in cultures of *Azotobacter* grown in N_2 cannot be regarded as critical evidence in favor of a view current that the ammonia observed is derived, either wholly or in any part, specifically and directly from N_2.

It is true that it is the reduced form of nitrogen which the organisms ultimately use to build protoplasm. It must be remembered, however, that the first product of nitrogen fixation will depend entirely on the catalysts which the organism has at its disposal and not on the form of nitrogen from which is built the protein complex of the cells.

Burk (*3*) discussed the mechanism of respiration of *Azotobacter* in the light of his data and from the point of view of chain kinetics and contact catalysis. He concluded that both oxygen and the glucose molecule must be in direct contact with the solid respiration catalyst if the reaction is to take place measurably, and that bombardments by unadsorbed molecules were ineffective.

From the fact that the rate of respiration of *Azotobacter* continues to increase with increasing partial pressures of oxygen, as shown by the data presented in this paper, it is clear that oxygen up to 1 atmosphere pressure does not inhibit the rate of its own consumption. It is evident from the data presented that the rate of respiration does not violate the mass law, as stated by Burk (*3, p. 1206*). Recalling that the rate of respiration is proportional to the partial pressure of oxygen, it appears that the reaction proceeds by bombardment and that direct contact of the reactants with a solid respiration enzyme is not imperative. It is also apparent that respiration is a first order reaction with respect to oxygen.

SUMMARY

The rate of respiration of *Azotobacter* during nitrogen fixation was studied over the entire range of oxygen pressures. The rate of respiration was found to be dependent on the partial pressure of oxygen provided the carbon dioxide is kept to a low value and the medium is kept saturated with oxygen at the partial pressure in question. Below 2 percent oxygen while fixing nitrogen *Azotobacter* respires with a respiratory quotient well above unity. At all concentrations of oxygen above 2 percent the heat measured was found to be equal to that calculated except at 78 percent. Here the heat measured was found to be approximately 57 percent of that calculated from the carbon dioxide evolved.

When *Azotobacter* was assimilating the ammonium ion the rate of respiration was found to be proportional to the concentration of oxygen from 5 to 80 percent. The heat measured was found to be equal, at all concentrations of oxygen above 5 percent, to that calculated from the carbon dioxide evolved. Below 5 percent intramolecular respiration was found to increase as the partial pressure of oxygen was decreased until at 0.1 percent oxygen the respiratory quotient reached 3.54.

The rate of respiration was found to be directly proportional to the concentration of oxygen from 10 to 100 percent when *Azotobacter* was allowed to assimilate the nitrate ion for its source of nitrogen. Below 10 percent oxygen intramolecular respiration occurred to a

considerable extent. The respiratory quotient reached 5.0 at 1 percent oxygen.

When the oxygen concentration is suddenly decreased from one-twentieth to one two-hundredth of that in air, considerable time is required to reduce the partial pressure of oxygen in the culture and within the cells to a point where the rate of respiration of the culture proceeded at a minimum constant rate.

Increasing the concentration of oxygen does not decrease the rate of its own consumption under the experimental conditions to which *Azotobacter* was subjected in these studies.

The data presented indicate that the first product of nitrogen fixation may be a union of nitrogen with oxygen.

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INHERITANCE OF SEED COLOR IN LACTUCA SATIVA[1]

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INTRODUCTION

The mature fruit of lettuce is an achene or naked seed, the color of which is determined by the color of its pericarp. The pigments that give the seed its particular color seem to be localized in the pericarp tissues.[2]

The mode of inheritance of seed color is of immediate practical importance to those breeding lettuce for disease resistance, climatic adaptation, and desirable commercial characteristics. Unlike flowers of most other food crops, those of lettuce can be emasculated only with extreme difficulty, if at all. In making crosses the breeder must remove the maternal parent's own pollen from the stigmas after the anthers have dehisced, but before the pollen tubes have entered the stigmatic tissues. As it is generally almost impossible to remove every grain of the maternal parent's pollen, both selfed and hybrid seed develop in heads in which pollen removal is attempted. Therefore, the breeder must know the mode of inheritance of some easily recognizable character by which he can distinguish hybrid from selfed plants in the F_1 generation. Seed color is a character that can be used to advantage for this purpose.

The cultivated varieties of lettuce (*Lactuca sativa* L.) may be roughly divided into 3 groups on the basis of their achene color. Of the 114 varieties of lettuce listed by Tracy [3] in 1904, 81 were indicated to have white, 30 black, and 3 yellow seed. Since then the trend has been even more toward the white-seeded varieties, until now there are only a few important varieties of the black-seeded type and no important varieties in the yellow-seeded group.

In the white-seeded group, the color is fairly constant except for discolorations caused by premature harvesting, dampness, and other environmental factors. In the black-seeded group the achenes range in color from reddish brown through dark brown to black. These different shades probably have a genetic basis; however, the differences are not great enough to permit accurate classification in view of the minor color variations caused by environmental factors. There are also some shade variations among varieties in the yellow group

Some data have been published by Durst [4] and Thompson [5] on the inheritance of factors for black and white seeds, but no report that deals with the factors for yellow seed has been found. The data of Durst and Thompson agree that black behaves as a monogenic factor

[1] Received for publication September 11, 1942.

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dominant to white. It is the purpose of this paper to show the relation of the factors that control the inheritance of the three colors, but no effort was made in this study to account for the inheritance of the minor color variations within the three large groups.

MATERIALS AND METHODS

The data presented were obtained from numerous hybrid populations of lettuce stocks being grown at the Bureau of Plant Industry Station, Beltsville, Md., for breeding heat-tolerant and disease-resistant varieties. The plants were grown either in 10-inch clay pots or in 1-foot by 2-foot by 3-inch cypress planting flats in a screened greenhouse. The possibility of contamination by insects was kept at a minimum by frequent spraying and fumigation.

The parent stocks had been grown for at least two generations previous to the cross from which seed-color records were kept and were known to be homozygous for the particular color type. The parent stocks used for the black phenotype were from a strain of Grand Rapids that has been maintained by the Division of Fruit and Vegetable Crops and Diseases for many years. For the white phenotype the variety Chavigne from Vilmorin-Andrieux & Cie., Paris, France; Iceberg from the Ferry-Morse Seed Co., San Francisco, Calif.; and a hybrid selection from a breeding stock were used. The variety Giant Summer from the Ferry-Morse Seed Co. was used for the yellow-seeded parent type.

In all cases the flower heads of the maternal parent were depollinated with water and dried before the desired pollen was applied by brushing the washed stigmas with the pollen-laden styles of flowers from the male parent.

GENETIC RELATIONS OF FACTORS FOR ACHENE COLOR

WORKING HYPOTHESIS

The breeding behavior of the three color types indicates that achene color in lettuce is inherited in a manner similar to that of the factors (*CcAa*) for coat color in rodents in which an F_2 generation from a cross between a black (*CCaa*) and an albino (*ccAA*) consists of 9 agouti, 3 black, and 4 albino.

This requires the presence of an independent allelomorphic pair in addition to those previously suggested by Durst [6] and Thompson [7]. In these two reports the genetic factor for black was represented by the symbol *WW* and the white by the symbol *ww*. This new pair of allelomorphs has been given the symbol *Yy*. The symbolized genotypes and their phenotypic expressions are *WWYY*, black; *wwYY*, yellow; *WWyy*, white; and *wwyy*, white. All four of these homozygous genotypes have been isolated, and their genetic constitution has been studied.

CROSS INVOLVING BLACK- AND YELLOW-SEEDED PARENTS

Grand Rapids × *Giant Summer* (*cross No. 122*).—Pollen from flowers of the yellow-seeded variety Giant Summer (*wwYY*) was applied to washed stigmas of flowers of the black-seeded variety Grand Rapids (*WWYY*). The cross was made in this way so that it might be pos-

[6] See footnote 4.
[7] See footnote 5.

sible to isolate at an early stage the hybrids from the selfed plants in the resulting progenies. Such isolation is possible because, as Thompson has shown,[8] the spotted anthocyanin leaf type of Giant Summer is dominant to the absence of pigment in the leaves of Grand Rapids.

A population of 93 F_2 plants was grown, with a ratio of 73 black-seeded to 20 yellow-seeded resulting. This is a satisfactory fit to a 3 to 1 ratio.

F_3 progenies were grown from both black- and yellow-seeded F_2 plants. Progenies from the F_2 yellow-seeded plants were all yellow-seeded. In the progenies from the black-seeded F_2 plants there were some families that were all black-seeded and other families with black- and yellow-seeded plants in the ratio of approximately 3 black-seeded to 1 yellow-seeded. The results from the F_2 and F_3 generations are given in tables 1 and 2, respectively.

TABLE 1.—*Records for F_2 progenies from selfed F_1 plants from crosses involving black-, yellow-, and white-seeded varieties of lettuce*

Phenotypes and cross	Parental varieties and assumed genotypes	F_1 plants	F_1 seed color	F_2 progenies				Assumed ratio	χ^2
				Black	Yellow	White	Total		
Black-seeded × yellow-seeded:		*Number*		*Number*	*Number*	*Number*	*Number*		
No. 122	Grand Rapids (*WWYY*) × Giant Summer (*wwYY*).	17	Black	73	20	0	93	3:1	[1] 0.5198
Black-seeded × white-seeded:									
No. 12	Grand Rapids (*WWYY*) × Iceberg (*WWyy*).	27	do	89	0	19	108	3:1	4.0000
No. 396	Grand Rapids (*WWYY*) × hybrid (*wwyy*).	35	do	88	42	52	182	9:3:4	[2] 4.5866
White-seeded × yellow-seeded:									
No. 21	Chavigne (*WWyy*) × Giant Summer (*wwYY*).	42	do	96	36	46	178	9:3:4	.4549
No. 40	Hybrid (*wwyy*) × Giant Summer (*wwYY*).	28	Yellow	0	42	13	55	3:1	.0958

[1] χ^2 for 1 degree of freedom (3:1 ratio) at 1-percent level, 6.635; at 5-percent level, 3.841.
[2] χ^2 for 2 degrees of freedom (9:3:4 ratio) at 1-percent level, 9.210; at 5-percent level, 5.991.

CROSSES INVOLVING BLACK- AND WHITE-SEEDED PARENTS

Grand Rapids × Iceberg (cross No. 12).—The white-seeded variety Iceberg (*WWyy*) was used as the pollen parent in a cross with the black-seeded Grand Rapids (*WWYY*). All the 27 F_1 plants grown to maturity produced black seed. The F_2 progenies segregated in the ratio of approximately 3 black- to 1 white-seeded. In the F_3 generation all the white-seeded F_2 plants gave families that produced only white seed. The black-seeded F_2 plants gave some families that were all black-seeded and other families that were black- and white-seeded in the ratio of approximately 3 black to 1 white. The F_2 and F_3 data are given in tables 1 and 2, respectively.

Grand Rapids × white-seeded hybrid (cross No. 396).—The white-seeded parent (*wwyy*) used in this cross was isolated from an F_3

[8] See footnote 5.

TABLE 2.—*Records for F_3 progenies from selfed F_2 plants from crosses involving black-, yellow-, and white-seeded varieties of lettuce*

Phenotypes and cross	Parental varieties and assumed genotypes	F_2 families	F_2 seed color	F_3 progenies				Assumed ratio	χ^2
				Black	Yellow	White	Total		
		Number		*Number*	*Number*	*Number*	*Number*		
Black-seeded × yellow-seeded:	Grand Rapids	6	Black	49	0	0	49		
No. 122	(*WWYY*)×Giant	11	do	62	15	0	77	3:1	[1] 1.1409
	Summer (*wwYY*)	3	Yellow	0	31	0	31		
Black-seeded × white-seeded:									
	Grand Rapids	9	Black	51	0	0	51		
No. 12	(*WWYY*) × Iceberg (*WWyy*).	15	do	75	0	20	95	3:1	.7895
		4	White	0	0	44	44		
		9	Black	87	0	0	87		
		10	do	71	28	0	99	3:1	.5690
	Grand Rapids	15	do	111	0	30	141	3:1	1.0425
No. 396	(*WWYY* × hybrid (*wwyy*).	37	do	197	71	96	364	9:3:4	[2] .6788
		17	Yellow	0	127	36	163	3:1	.7383
		5	do	0	47	0	47		
		27	White	0	0	259	259		
White-seeded × yellow-seeded:									
		6	Black	56	0	0	56		
		18	do	126	47	0	173	3:1	.4952
	Chavigne (*WWyy*)	13	do	98	0	29	127	3:1	.3760
No. 21	× Giant Summer (*wwYY*).	29	do	151	50	80	281	9:3:4	2.0405
		11	Yellow	0	91	17	108	3:1	4.9382
		8	do	0	77	0	77		
		35	White	0	0	341	341		
	Hybrid (*wwyy*) ×	3	Yellow	0	29	0	29		
No. 40	Giant Summer	12	do	0	70	17	87	3:1	1.5209
	(*wwYY*).	5	White	0	0	35	35		

[1] χ^2 for 1 degree of freedom (3:1 ratio) at 1-percent level, 6.635; at 5-percent level, 3.841.
[2] χ^2 for 2 degrees of freedom (9:3:4 ratio) at 1-percent level, 9.210; at 5-percent level, 5.991.

population of the cross No. 21 between the white-seeded Chavigne and the yellow-seeded Giant Summer. As indicated in the discussion of cross No. 21, one-fourth of the F_2 plants produced white seed. According to the hypothesis, one-fourth of these F_2 white-seeded plants should be of the double-recessive (*wwyy*) genotype. Seven of these F_2 white-seeded segregates were tested by backcrossing to the yellow-seeded Giant Summer, and one of these was found to be of the double-recessive genotype, as indicated by an all yellow-seeded F_1 generation and an F_2 generation that gave 3 yellow-seeded to 1 white-seeded. This double recessive was then crossed with Grand Rapids (*WWYY*). The F_1 plants of this cross were black-seeded, and the F_2 segregated approximately 9 black-seeded, 3 yellow-seeded, and 4 white-seeded.

The F_3 progenies from the white-seeded F_2 plants were all white-seeded. Some progenies from the black-seeded F_2 plants produced only the black-seeded type; some produced 3 black-seeded to 1 yellow-seeded; some 9 black-seeded, 3 yellow-seeded, and 4 white-seeded; and some 3 black-seeded to 1 white-seeded. Some progenies of yellow-seeded F_2 plants produced all yellow-seeded F_3 and others 3 yellow-seeded to 1 white-seeded. The F_2 and F_3 data are given in tables 1 and 2, respectively.

CROSSES INVOLVING YELLOW- AND WHITE-SEEDED PARENTS

Chavigne × Giant Summer (cross No. 21).—The yellow-seeded variety Giant Summer (*wwYY*) was used as the pollen parent in a cross with the white-seeded variety Chavigne (*WWyy*). The F_1

hybrid plants all produced black seed. The F_2 population segregated 9 black-seeded, 3 yellow-seeded, and 4 white-seeded. The F_3 progenies from white-seeded F_2 plants gave only white seed. The F_3 progenies from yellow-seeded F_2 plants gave some families that were all yellow-seeded and other families that segregated with a moderately significant departure from 3 yellow- to 1 white-seeded. F_3 progenies from black-seeded F_2 plants were all black-seeded, 3 black-seeded to 1 yellow-seeded, 3 black-seeded to 1 white-seeded, or 9 black-seeded to 3 yellow-seeded to 4 white-seeded. The F_2 and F_3 data are presented in tables 1 and 2, respectively.

White-seeded hybrid × *Giant Summer* (*cross No. 40*).—The yellow-seeded Giant Summer (*wwYY*) was used as the pollen parent in a backcross to the white-seeded F_3 selection (*wwyy*) from Chavigne × Giant Summer. The F_1 backcrossed plants all produced yellow seed, and the F_2 population from these segregated 3 yellow- to 1 white-seeded. The F_3 progenies from the white-seeded F_2 plants were all white-seeded. From the F_2 yellow-seeded plants some families that were all yellow-seeded and other families that segregated 3 yellow-seeded to 1 white-seeded were obtained. F_2 and F_3 data are presented in tables 1 and 2, respectively.

DISCUSSION

The 3 to 1 ratio of black to white seed in the F_2 generation, as suggested by Durst [9] and by Thompson,[10] holds only in the case of the white-seeded phenotype having the *wwYY* constitution. With the double-recessive white-seeded (*wwyy*) the F_2 from a cross with a black-seeded variety gives the ratio of 9 black-seeded, 3 yellow-seeded, to 4 white-seeded.

Many varieties of lettuce of the cultivated form (*Lactuca sativa*) have come under the writer's observation in the lettuce-breeding program of the United States Department of Agriculture, and breeding data on seed color have been recorded on many of these. None of the white-seeded varieties on which breeding behavior records are available are of the double-recessive (*wwyy*) type. This type must be very rare among fixed varieties.

SUMMARY

Data from the F_1 generation and from F_2 and F_3 progenies of crosses involving the three seed-color types in lettuce—black, yellow, and white—are presented.

The data have been analyzed for an explanation of the inheritance of the factors that control pericarp color in lettuce seed. The analysis indicates that the expression of black, yellow, and white seed color in cultivated lettuce (*Lactuca sativa*) is controlled by two pairs of allelomorphs, which have been assigned the symbols *Ww* and *Yy*. The inheritance is similar to that of the factors (*CcAa*) for coat color in rodents in which the F_2 from a cross between black and albino parents gives 9 agouti, 3 black, and 4 albino. In the present case the F_2 progenies from a cross between a white-seeded (*WWyy*) and the yellow-seeded (*wwYY*) segregated 9 black-seeded, 3 yellow-seeded, and 4 white-

[9] See footnote 4.
[10] See footnote 5.

seeded. This gives one true-breeding black genotype (*WWYY*), one true-breeding yellow genotype (*wwYY*), and two true-breeding white genotypes (*WWyy*) and (*wwyy*).

The double-recessive white-seeded genotype (*wwyy*) is rare, if present at all, among commercial varieties of lettuce. A homozygous line of this genotype was obtained only by the use of testers on the white-seeded segregates from a yellow-seeded by a white-seeded cross in which the white-seeded was of the *WWyy* genotype.

INHERITANCE OF OIL GLANDS IN PIMA COTTON [1]

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INTRODUCTION

Small spherical oil glands occur in cotton, on the herbage, flowers, and bolls, usually more or less embedded in the surrounding tissue. Such glands are apparently universal in the genus *Gossypium* L., according to Watt [2] and other authors. Some data pertaining to the inheritance of oil glands in Pima cotton, an agronomic variety of *G. barbadense* L., are presented in the following account. Although taxonomists have utilized differences in characteristics of oil glands, the writers are not aware of any previous genetic study of these bodies in the genus *Gossypium*.

Smith [3] applied the designation "P Hope" to a family of Pima cotton that is characterized by smooth bolls. Smith found that "pitted" and "smooth" are inherited in monohybrid fashion, and for these characters proposed the symbols B^p and B^s, respectively. Family P Hope also has three other distinguishing characters, namely, delayed development of oil glands on the young bolls, smaller and more deeply embedded glands on the stem, and smaller glands on the calyx. The characters that distinguish P Hope from ordinary Pima cotton are described in the following section.

DESCRIPTION OF CHARACTERS

DEVELOPMENT OF BOLL GLANDS

In family P Hope, the oil glands are wanting, or at least not discernible, until the third day after anthesis. The character is termed "late." In PH8 and other families of the Pima variety that have pitted bolls, the normal condition in *Gossypium barbadense*,[4] the boll glands are "early," that is, conspicuously evident on the day following anthesis. In fact, pigmented oil glands occur on the ovary of PH8 flower buds. The aspect of bolls of P Hope, PH8, and PH8 × P Hope F_1, on the first day after anthesis, is illustrated in figure 1.

STEM GLANDS

Oil glands on the stem, or main axis, of P Hope differ in size from corresponding glands of family PH8. Although stem glands are actually as numerous in P Hope as in PH8 (table 1), the glands are deeply embedded and consequently less conspicuous.

[1] Received for publication November 13, 1942.

[2] WATT, SIR GEORGE. THE WILD AND CULTIVATED COTTON PLANTS OF THE WORLD. 406 pp., illus. London. 1907.

[3] SMITH, E. G. INHERITANCE OF SMOOTH AND PITTED BOLLS IN PIMA COTTON. Jour. Agr. Res. 64: 101-103, illus. 1942.

[4] Early development of the boll glands is apparently the rule also in upland cotton (*Gossypium hirsutum* L.), in which the bolls are not pitted. Glandless bolls sometimes occur in Hopi cotton (*G. hopi* Lewton) and in some segregates of crosses involving Hopi cotton, but in these cases the glandless condition persists through maturity of the bolls.

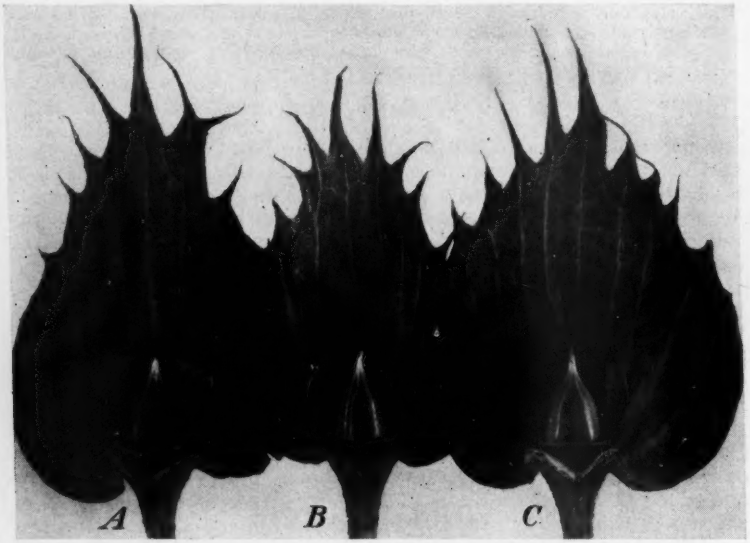

FIGURE 1.—Aspect of bolls on the first day after anthesis. The glands are numerous and conspicuous in (*A*) family PH8, comparatively sparse in (*B*) PH8 × P Hope F_1, and wanting, or at least not discernible, in (*C*) family P Hope. ×1.4.

CALYX GLANDS

The calyx glands of family P Hope, like the stem glands, are smaller than the corresponding glands of family PH8. The difference in size, although not large, is sufficient to make feasible the classification of nearly all plants in segregating populations. The relative size of the oil glands in PH8, P Hope, and PH8 × P Hope F_1 is observable in the calyxes shown in figure 1. Calyx glands are also illustrated, at greater magnification, in figure 2.

Calyx and stem glands are classified as of large, intermediate, or small size.

BOLL SURFACE

Pitted boll surface is the condition found in normal Pima cotton. The surface is dotted with conspicuous pits, and many of the oil glands are large and shallowly embedded. In family P Hope, the bolls may be described as "smooth," the surface being obscurely pitted and the oil glands small and deeply embedded (fig. 2).

As Harland [5] (*pp. 117–118*) points out, in the Peruvian group of cottons, to which the Pima variety belongs, the oil glands are near the surface, and around some of them the surface is depressed in small craterlike pits. Pits do not occur when the glands are deeply embedded, as in the Pima family P Hope and in upland cotton (*Gossypium hirsutum* L.). Therefore, it can be seen that a very close relation exists between the pitting of the boll surface and the depth at which the oil glands are embedded in the tissue.

[5] HARLAND, S. C. THE GENETICS OF COTTON. 193 pp., illus. London. 1939.

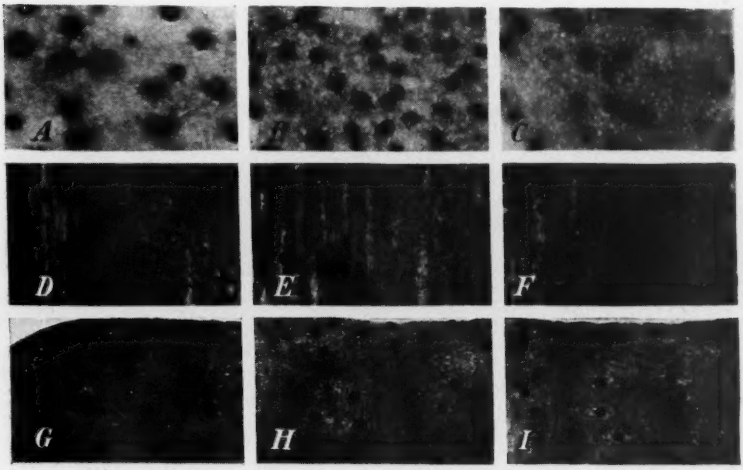

FIGURE 2.—Oil glands of bolls, stems, and calyxes in Pima cotton. Boll glands in (*A*) family PH8, (*B*) PH8 × P Hope F_1, and (*C*) family P Hope; stem glands in (*D*) PH8, (*E*) PH8 × P Hope F_1, and (*F*) P Hope; calyx glands in (*G*) PH8, (*H*) PH8 × P Hope F_1, and (*I*) P Hope. ×11.

TABLE 1.—*Classification of populations grown in 1941 in respect to character o boll surface and the mean number of oil glands in an area of 25 sq. mm. on the bolls and stems*

Population designation	Classification in respect to boll surface[1]	Number of plants	Number of oil glands per unit area			
			On bolls		On stems	
			Mean	Standard error of mean	Mean	Standard error of mean
PH8	Pitted	13	25.1	0.67	33.4	2.11
P Hope	Smooth	18	22.3	1.06	35.8	1.63
PH8 × P Hope F_1	Intermediate	17	46.4	1.23	35.9	1.50
PH8 × P Hope F_2	Pitted	10	31.2	1.62	40.8	1.34
Do	Intermediate	20	43.3	1.19	34.7	1.49
Do	Smooth	6	20.2	2.14	34.3	3.25
(PH8 × P Hope F_1) × PH8 F_1	Pitted	20	28.7	.78	39.4	1.54
Do	Intermediate	21	45.9	1.23	37.0	1.72
(PH8 × P Hope F_1) × P Hope F_1	Smooth	6	23.7	2.11	37.5	3.55
Do	Intermediate	14	48.3	1.84	38.6	1.90

[1] The three oil gland characters mentioned in the text do not segregate independently of boll surface. Therefore, in this column pitted boll surface and smooth boll surface also denote, respectively, early and late development of boll glands, large and small stem glands, and large and small calyx glands. Individuals intermediate for boll surface are also intermediate for the other characters.

In the characters described above, the F_1 is intermediate between the two parental types. This statement does not apply, however, to the number of boll glands, which will be discussed later

METHODS

All individuals in the several populations were classified independently for each character. Repeated observations on the stem

and calyx glands, particularly the latter, were required in order to ascertain the true classification of the plants.

Boll glands were counted in an area of 25 sq. mm. located in the center of a valve. Stem glands were counted in an area of the same size located in the middle of the seventh internode below the apex of the main stem. On each plant five boll counts and five stem counts were made, and as many calyx counts as possible, depending on the number of flowers available. Calyx glands were counted in a strip 5 mm. wide, extending from the upper edge of the inner nectary to the rim of the calyx, an area that averaged 41 sq. mm. The use of a low-power dissecting microscope and transmitted light brought to view deeply embedded glands that ordinarily are not visible. In the case of the bolls, the thickness of the wall necessitated removal of some of the soft inner tissue in order to allow passage of sufficient light to illuminate deeply embedded glands.

RESULTS

In 1940, in an F_2 population of 53 individuals that segregated in a 1:2:1 ratio in respect to pitted, intermediate, and smooth boll surface (Smith, table 1),[6] it was observed that the three gland characters mentioned above did not segregate independently of boll surface. The material grown in 1941 enabled the writers to make further observations on the relation of the oil gland characters to the character of the boll surface.

No clear case of dissociation appeared in any of the 97 individuals grown in the F_2 and backcross populations in 1941. Owing to poor development, 4 plants could not be classified satisfactorily in respect to stem glands. More difficulty, possibly resulting from the presence of modifying factors, was encountered in classifying calyx glands, and at the end of the season 11 plants still remained in doubtful status. However, in no instance was a plant classified with certainty as having any combination of the 4 characters other than those that exist in one or the other parent family or in F_1. Therefore, in the absence of positive evidence to the contrary, the 97 individuals in F_2 and backcross populations were classified (table 1) as follows: In 55 plants the 4 characters were intermediate, in 30 plants the characters were associated as in family PH8, and in 12 plants as in family P Hope.

A phenomenal increase in number of boll glands occurs in F_1 and in heterozygous plants in other populations. The mean number of oil glands per unit area in F_1 is 46.4, or approximately the sum of the means of PH8 and P Hope (table 1). Somewhat similar results were obtained in 1940, the counts having been 47.0 for plants with intermediate boll surface, 27.5 for plants with pitted bolls, and 26.3 for smooth-boll individuals. The relatively large number of boll glands in the F_1 is clearly shown in figure 2 (*B*, as compared with *A* and *C*).

The range in number of boll glands per unit area, for each class of all the 1941 populations combined as one array, is as follows: 14 to 29 for plants with smooth bolls, 34 to 58 for heterozygous plants with intermediate bolls, and 23 to 39 for those of the pitted-boll class. The mean number of boll glands in family PH8 exceeds the mean in family P Hope by 2.8 glands (table 1). This difference is only 2.2 times its standard error. However, in view of the ranges in boll

[6] See footnote 3, p. 447.

gland number noted above, the chances are that the smaller number of glands observed in plants having smooth bolls is inherent rather than fortuitous.

The great increase in boll gland number in the heterozygotes is an unusual manifestation of heterozygosity, especially in view of the fact that heterosis is not otherwise apparent in this cross between families that are similar in all but the gland characters. The large number of glands in heterozygous individuals may be explained by assuming that the genes B^p and B^s are complementary in respect to number of boll glands, either gene alone giving rise to only half the number of glands that appear when the two genes are brought together.[7]

The mean number of calyx glands per unit area was found to be 99.9 ± 2.30 in family PH8, 99.0 ± 1.65 in family P Hope, and 96.8 ± 1.54 in PH8×P Hope F_1. Since these populations differed so slightly in regard to number of calyx glands, counts were not made in other populations.

The mean number of stem glands per unit area is given, for each population, in table 1. This character seems unimportant, considering the very small difference (2.4 ± 2.67) between the means of the parent families and the almost identical means in P Hope and PH8×P Hope F_1.

The ratios obtained by Smith (table 1)[8] clearly indicate monohybrid inheritance of smooth and pitted boll surface. The ratios obtained in 1941 are disturbed, but not too seriously, by deficiency of smooth-boll individuals. The values of χ^2 and P given in table 2 are computed from data in table 1. The probability values do not indicate significant departure from the calculated monohybrid ratios.

CONCLUSIONS

The unbroken association of the characters (boll surface, development of the boll glands, size of the stem glands, and size of the calyx glands) that occur only in the combinations existing in the parent families and in F_1, is either an instance of complete linkage or, more probably, a case in which a single gene conditions several phenotypic characters.

TABLE 2.—*Values of χ^2 and P, computed from data in table 1, for the F_2 and backcross populations grown in 1941*

Population designation	X^2	P [1]
PH8 × P Hope F_2	1.33	0.5
(PH8 × P Hope F_1) × PH8 F_1	.02	.9
(PH8 × P Hope F_1) × P Hope F_1	3.20	.1

[1] The probability (P) values do not indicate significant deviation from calculated monohybrid ratios.

Indisputable evidence of linkage in the genus *Gossypium* appears to be scanty. Harland (*p. 159*)[9] recognizes only three clear cases. Since Harland wrote, McMichael [10] has reported complete association

[7] Acknowledgement is made to J. H. Kempton, formerly of the Division of Cereal Crops and Diseases, Bureau of Plant Industry, for having suggested this explanation.

[8] See footnote 3, p. 447.

[9] See footnote 5, p. 448.

[10] McMICHAEL, S. C. OCCURRENCE OF THE DWARF-RED CHARACTER IN UPLAND COTTON. Jour. Agr. Res, 64: 477–481. 1942.

of dwarfness and red plant color in Acala cotton, an agronomic variety of *G. hirsutum*. McMichael mentions the possibility of linkage, but nevertheless concludes his account with the following statement: "Indications are that dwarf-red is controlled by a single factor."

In view of the few well-established instances of linkage in cotton, the probability is that the characters involved in the present study are merely different manifestations of a single pair of genes. This conclusion is strengthened by the fact that all of the characters (indirectly, in the case of boll surface) pertain to oil glands.

SUMMARY

Smooth boll surface and three previously undescribed oil gland characters, namely, delayed development of the boll glands, small and deeply embedded stem glands, and small calyx glands, characterize a family of Pima cotton (*Gossypium barbadense* L.) known as P Hope. The contrasting characters, namely, pitted boll surface, early development of the boll glands, large and shallowly embedded stem glands, and large calyx glands, are found in PH8, a representative family of normal Pima cotton.

Observations made in segregating populations of the cross PH8 × P Hope strongly indicate that the characters mentioned above do not segregate independently but, on the contrary, occur only in the combinations existing in the parent families and in F_1. This association of the characters is tentatively accounted for as an instance of manifold expression of a single pair of genes rather than as complete linkage.

Ratios obtained in F_2 and backcross populations agree fairly well with expectations based on the assumption of a single-factor difference. Monohybrid inheritance previously had been found to be the mode of inheritance of pitted boll surface and smooth boll surface, for which the symbols are respectively B^p and B^s. All the above-mentioned characters are intermediate in F_1.

A remarkable increase in number of boll glands occurs in F_1, also in the heterozygous class in F_2 and backcross populations. The mean number of boll glands in F_1 nearly equals the sum of the means that obtain in family PH8 and family P Hope. This is a noteworthy manifestation of heterozygosity, which may be explained by assuming that the genes B^p and B^s are complementary in respect to number of boll glands, either gene alone giving rise to only half the number of glands that appear when the two genes are brought together in the heterozygote.

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